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STUDY OF THE TULAREMIA PATHOGEN-SIMILAR V. NOVICIDA LARSON ET AL MICROORGANISM

[Following is the translation of an article by N. G. Olsuf'yev, O. S. Yemel'yanova, I. S. Meshcheryakova, and I. V. Rodionova, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology, and Immunobiology) No 8, 1968, pages 92-98. It was submitted on 29 Dec 1967.]

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In 1955 Larson and associates in the USA described a new microorganism - Pasteurella novicida - which is very similar with the causative agent of tularemia. A unique strain of this microbe was isolated during passaging of a sample of water through the organism of guinea pigs and white mice. The water was obtained from the Ogden Bay Bird Reservation on the Great Salt Lake in the state of Utah. This strain differed from the tularemia causative agent mainly by the absence of cross serological reactions, the ability to ferment saccharose, and a somewhat lesser pathogenicity for white mice and guinea pigs. Olsuf'yev and associates (1959), and then Owen and associates (1964), related this microbe to the genus Francisella. Numerous attempts to detect F. novicida at the site of the initial isolation and in other places in the USA turned out to be unsuccessful.

Thanks to the kindness of Doctor K. Owen (Rocky Mountain Laboratory, Hamilton, USA) and Professor Mollare (Pasteur Institute, Paris, France), to whom we express our thanks, we obtained an original strain of F. novicida and studied it. Below we present the results of the investigation of the culture obtained from Doctor Owen. We propose that knowledge of these investigations will be useful for Soviet microbiologists, since it is not excluded that this microorganism may be detected in the USSR.

Based on morphology cells of F. novicida are similar to the tularemia causative agent, in particular in a culture on coagulated vitelline medium; on blood agar with cystine and glucose they are somewhat larger. F. novicida, just as the tularemia microbe, gives off mucus. The considerable similarity, revealed during an electron-microscopic investigation of them, with the tularemia microbe is relative mainly to the structure of the cell wall, which has a smooth-contour outer membrane, and also the capacity for division by means

of budding (I. B. Pavlova and I. S. Meshcheryakova, personal communication).

On coagulated yolk medium and blood agar the studied strain grew somewhat more flourishing, forming larger colonies than the tularemia causative agent. Following mass seeding in meat-peptone broth growth was exceptionally weak (100-200 million based on the turbidity standard of the Tarasevich Institute), on meat-peptone agar individual fine colonies grew, and following reseeding growth ceased. When F. novicida was seeded on Down's medium with glycerin a clear yellowing of the medium was noted (glycerin). On Down's medium with saccharose fermentation was absent (for a control we used the Nearctic tularemia strains 38 and 0-284, which also did not ferment saccharose). We determined the oxidation capacity for saccharose more accurately by the direct manometric method of Warburg. It was established that the activity of respiration in F. novicida due to this carbohydrate comprised 100.73 microliters per 1 gram of nitrogen in the suspension of live cells during one hour of incubation. This testified to the moderate assimilation of the given substrate. In a control investigation of two Palaearctic tularemia strains 9 and 503 no activity was revealed relative to saccharose; an attempt at induction of the corresponding enzymatic systems with the subsequent reseeding of these strains on a medium which contained saccharose as the source of carbon did not prodube an effect even after 30 generations. We did not conduct an investigation of Nearctic strains in such a manner.

The activity of citrullinureidase in acetone-treated cells of F. novicida comprised 10.6 micromoles of ornithine per 20 mg of preparation for 3 hours of incubation, which corresponded to the level of activity of Nearctic races of tularemia microbes.

Pathogenicity for laboratory animals was determined on white mice (weight 22-25 g), guinea pigs (weight 200-250 g), and domestic rabbits (weight 2600-3000 g) by the subcutaneous administration of a 2-day culture from coagulated yolk medium (Table 1).

White mice died in 8-9 days, guinea pigs in 10-13 days, and a rabbit died in 3 days.

Autopsy revealed a number of pathological anatomical changes in the animals.

In mice the vessels of the subcutaneous cellular tissue were hyperemic and at the site of infection an infiltrate was usually absent. The spleen was enlarged and slightly consolidated. The liver was enlarged, not consolidated, and in a number of cases contained necrotic sectors. Visible changes were not detected in the lungs and intestines.

Table 1

Virulence of F. novicida for laboratory animals (following subcutaneous administration)

(a) Доза (в микробных клетках)	Результат (b)		
	белые мыши (c)	морские свинки (d)	домашние кролики (e)
1	1/3	1/3	
10	1/3	1/3	
100	0/3	2/3	
1 000	2/3	1/3	
10 млн. (f)			0/2
100 млн. (f)			0/2
1 млрд. (g)			1/2

Legend: numerator - number of animals which died; denominator - number of animals in test.
Key: (a) Dose (in microbial cells); (b) Result; (c) White mice; (d) Guinea pigs; (e) Domestic rabbits; (f) million; (g) billion.

In guinea pigs the vessels of the subcutaneous cellular tissue were hyperemic and at sites of infection in some cases there was an insignificant infiltrate. Inguinal lymph nodes were enlarged and hyperemic. The spleen was enlarged and contained necrotic nodules. The liver was enlarged and contained necrotic foci. In the lungs there were sectors of hyperemia and in certain cases - necrotic nodes.

In the rabbit the vessels of the subcutaneous cellular tissue were sharply hyperemic and there was a very significant infiltrate at the site of infection. The spleen was slightly enlarged, had a cherry color, but was not consolidated. The liver was enlarged sharply, had a light cherry color, and was dense. The lungs had sectors of hyperemia. There was hemorrhagic exudate in the chest and the abdominal cavity.

In smear imprints from the spleen and blood of dead white mice F. novicida either were not detected or individual groups of bacteria were noted in the spleen, but not in the blood. When fragments of spleen were seeded on nutrient medium the growth of the causative agent was always observed. In guinea pigs microbes were revealed only in seedings on coagulated yolk medium or blood agar (in some cases with the addition of penicillin). Results of bacterioscopy of smear imprints from the organs of the dead rabbit were also negative; a culture was obtained by sedding a fragment of lungs on blood agar.

For the purpose of determining immunological bonds with the tularemia microbe the surviving animals were infected under the skin with virulent strains of the tularemia microbe. White mice and guinea pigs were infected with 100 Dolm (100 microbial cells) of strain 503 of the Palaearctic race, and the rabbits - with 10 microbial cells of the Nearctic Schu strain. Without exception all the animals died from tularemia in periods which were somewhat extended in comparison with the periods of death of control animals. Consequently cross immunity was not detected.

Serological properties of F. novicida were studied in the reactions of agglutination, passive hemagglutination, and antibody neutralization. For the agglutination reaction we used a formalin-killed culture of the test strain, for the passive hemagglutination reaction - the thermoextract from bacteria as the sensitizing agent for adsorption on erythrocytes, and for the neutralization reaction - a formalin-inactivated culture of the test strain, corresponding antiserum, and erythrocytes which are sensitized by one or the other antigen. Agglutination and hemagglutination titers of sera were determined based on the last dilution in which a clear reaction was observed (no less than+++).

In 5 rabbits, which had endured infection by F. novicida, on the 15th day after subcutaneous infection with doses of 10 million - 1 billion microbial cells the agglutination titer of serum with homologous strain was equal to 1:160--1:640 and hemagglutination - 1:2560--1:40960; with the heterologous strain of tularemia microbe the agglutination titer comprised correspondingly 1:40--1:320, and hemagglutination - 1:80--1:1280. The higher the infection dose the higher the level of titers reached, and in all cases the reaction with homologous strain was higher than with heterologous. In guinea pigs which had endured infection by F. novicida the titers with homologous antigen equaled 1:80--1:160, but not one of these sera reacted with the tularemia causative agent (in a minimum dilution of 1:10).

In two rabbits which had endured infection following the subcutaneous administration of 100 million microbial cells, after a month a check was made of allergic reactivity to the intracutaneous administration of antigens from homologous and heterologous strains. Antigen from the test strain was prepared by the method used for the preparation of tularin; initial concentration of bacteria comprised 1 billion microbial cells in 1 ml (according to the standard of the Tarasevich Institute). We also used fractional doses of antigen, for which the initial concentration of bacteria was diluted by 10, 100, and 1000 times. The suspension was introduced in a volume of 0.1 ml, i.e., the doses introduced comprised 100 million, 10 million, 1 million, and 100,000 microbial cells. In rabbits which had endured F. novicida infection the allergic reactivity of the skin to homologous and heterologous antigens turned out to be more or less similarly moderately expressed (Table 2). In a control

healthy rabbit antigen of the test strain or tularin was administered in a dose of 100 million microbial cells and after 24 hours only a weak hyperemia of the skin without an infiltrate was noted. It disappeared in 48 hours.

Table 2

Results of testing the allergic reactivity of F. novicida

(a) Антиген	(b) Доза	Реакция (c) через 24 часа (d)	через 48 часов (e)	в последующие дни (f)
F. novicida	100 млн. (k)	Гиперемия и инфильтрат до 1 см (h)	Аналогичная (q)	Отсутствовала (r)
	10 млн. (i) Меньше (j)	То же (l)	Отсутствовала (m)	
(g) Туларин	100 млн. (k)	Гиперемия и инфильтрат до 1.5 см (n)	Аналогичная (q)	Отсутствовала (r)
	10 млн. (i) Меньше (j)	То же (o)	Отсутствовала (p)	

Key: (a) Antigen; (b) Dose; (c) Reaction; (d) in 24 hours; (e) in 48 hours; (f) on subsequent days; (g) Tularin; (h) 100 million; (i) 10 million; (j) Less; (k) Hyperemia and infiltrate up to 1 cm; (l) Same; (m) Absent; (n) Hyperemia and infiltrate up to 1.5 cm; (o) Same; (p) Absent; (q) Analogous; (r) Absent.

In 4 rabbits which had had tularemia after the intravenous administration of 1000 microbial cells of the virulent Palaearctic strain 503, in 10-15 days after infection the blood serum contained agglutinating titers of 1:460--1:2560 to the inherent strain and hemagglutinating - 1:20480--1:81920, while to the test strain the titers comprised correspondingly 1:10--1:80 and 1:320--1:2560, i.e., turned out to be many times lower than homologous. The nature of agglutination with both cultures was similar.

Cross cutaneous allergic reactions were also studied in rabbits which were hyperimmunized against tularemia (vaccination and subsequent infection with the Schu strain). In 24 hours the rabbits reacted with the development of an intense cutaneous reaction in the form of hyperemia and an infiltrate (up to 1-2 cm in diameter)

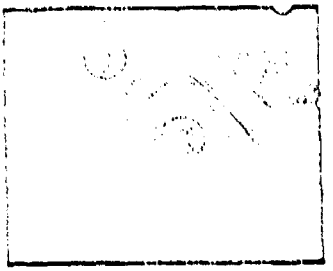
to the administration of tularin in doses of 10 and 1 million microbial cells and somewhat more weakly (diameter of sector 0.5-1 cm) - to the administration of 100,000 microbial cells; in 48 hours the intensity of the reaction was not reduced, but on subsequent days the reaction faded. The rabbits reacted to the administration of antigen from F. novicida almost with the same intensity - in 24 hours after administration of doses of 10 and 1 million microbial cells hyperemia and an infiltrate up to 1.5 cm in diameter were formed, and with doses of 100,000 microbial cells - up to 1 cm in diameter. After 48 hours the reaction preserved almost the same intensity and faded in subsequent days. It is necessary to note that hyperemia of the skin was somewhat more clearer for the tularemia antigen than for the antigen from F. novicida.

The result of the reaction of antibody neutralization with the test strain and the standard horse antitularemia serum, the hemagglutination titer of which equaled 1:160,000, was negative. In the testing of formalinized suspensions of F. novicida the neutralization of antibodies was not revealed even at a concentration equal to 1 billion microbial cells in 1 ml of suspension, while a homologous tularemia culture caused the neutralization of antibodies in a concentration equal to 2 million microbial cells. When the reaction was set up with tularemia microbes and serum against F. novicida, the hemagglutination titer of which equaled 1:5120 with homologous antigen, neutralization was absent. Formalinized cells of F. novicida inhibited the reaction of passive hemagglutination at a minimum concentration of 500,000 microbial cells, while tularemia causative agents did not inhibit the reaction even in a concentration equal to 1 billion microbial cells. These data testified to the diverse nature of species specific antigens of F. tularensis and F. novicida.

A comparative study of the antigenic structure of these microbes by the method of precipitation in gel (Ochterlony method) using the dry acetone powders of both strains in doses of 25 mg per 1 ml and standard horse antitularemia serum showed that these microorganisms had at least two common antigenic components contained in the somatic portion of the antigen complex. The presence of species specific Vi-antigen of F. tularensis in cells of F. novicida was not revealed (see drawing).

It is known that the tularemia microbe, in contrast to many other species of microbes, is lyzed in a twine solution (D. I. Brikman, personal communication). We used this test in the study of F. novicida while using 6 tularemia strains for a control: one strain of the Nearctic race - Schu, one - Central Asian 117, and 4 Palaearctic strains 503, 21/400, Tun Lyao, and Kosho. The Palaearctic strains differed in the degree of virulence. In addition to this an investigation was made of one strain of S. typhimurium and one of L. monocytogenes. Initial density of the suspension was around 1.5--3 billion in 1 ml. Determination of turbidity was

carried out by the photometric method and expressed in billions of cells by means of a comparison with a calibration curve. For construction of the curve we determined in a suspension of strain 503 the number of microbial cells in 1 ml based on the standard of the State Control Institute and took a photometric reading of them at 540 nm on a FEK-56. We investigated the suspensions immediately after preparation and also in 4 and 24 hours.



Reaction of precipitation in agar by the Ochterlony method. 1 - antigen of F. novicida; 2 - antigen of F. tularensis; 3 - standard horse antitularemia serum; thin lines - precipitation of somatic antigen complex, wide lines - precipitation of Vi-antigen complex of tularemia microbe, absent in F. novicida.

All the tularemia strains were lyzed in 4 hours and turbidity was reduced 1½--2½ times in comparison with a control suspension in a physiological solution. In contrast to this, a suspension of F. novicida in a twine solution after 4 hours of exposure differed very little in turbidity from a suspension in physiological solution. Analogous results were obtained with suspensions of Salmonella and Listeria. In 24 hours the suspension of the test strain was lyzed in a twine solution approximately up to the level of tularemia strains, while Salmonella and Listeria, after 24 hours, remained the same in twine solution as in physiological solution. In the investigation of Gram-stained smears from these cultures after 24 hours in twine it was not possible to reveal any morphological differences in them in comparison with cultures in physiological solution. After 24 hours in twine cells of the test strain and of tularemia causative agents were stained weakly and had vague contours, while in physiological solution they stained brightly and preserved sharp contours.

The results of our investigations confirm the findings of

Larson and associates and Owen and associates concerning the high degree of similarity in the morphological, tinctorial, cultural, and, in particular, pathogenic properties of F. novicida and tularensis. In agreement with the findings of these authors we also detected in F. novicida the capacity to ferment saccharose, which is not a characteristic of the tularemia causative agent.

However, in F. novicida and tularensis we established a partial antigenic community by means of using antisera from rabbits in the agglutination reaction, which the investigators mentioned could not demonstrate. They observed partial cross reactions only in tests of complement fixation.

Once more we established the antigenic nearness of both microorganisms in the allergy reaction on rabbits, detected the enzyme citrullinureidase in F. novicida, and demonstrated the weaker lyzability of this microorganism by twine.

Under practical conditions for the diagnostics of F. novicida, apart from establishing the close similarity of main properties of the isolated culture with the tularemia causative agent, it is necessary to pay special attention to the weak agglutinability of the culture by standard agglutinating antitularemia serum (no more than up to 1/30 the titer of the latter) and to the fermentation of glycerin on Down's medium. The last criterion can serve for differentiation only with tularemia cultures of the Palaearctic race, but not of the Central Asian, Nearctic, or Japanese variants of the Palaearctic race which ferment glycerin.

When cultures which are suspected of being F. novicida are isolated from water or other objects they should be sent for confirmation to the Gamaleya Institute of Epidemiology and Microbiology, AMN USSR (Moscow, Ulitsa Galaleya 2).

Conclusions

1. Francisella novicida, which was described by Larson and associates, based on its morphological, tinctorial, cultural, and, in particular, pathogenic properties for laboratory animals turned out to be similar to the causative agent of tularemia, which is the basis for combining both microorganisms in one genus.

2. F. novicida decomposed glycerin and contained the enzyme citrullinureidase, which connected this species with the Nearctic and Central Asian variants of the tularemia microbe.

3. F. novicida fermented saccharose and was lyzed relatively slowly in a twine solution, by which it differed from the causative agent of tularemia.

4. In F. novicida we detected a community of the somatic antigenic complex with the corresponding complex of the tularemia microbe, which in tests on rabbits conditioned the partial cross reactions of agglutination and allergy.

5. On laboratory animals a live culture of F. novicida did not guarantee protection against repeated infection with lethal doses of a virulent tularemia culture.

6. On the territory of the USSR it is necessary to organize searches for F. novicida primarily in reservoirs - in places of mass congregation of birds, and also on the birds themselves and rodents living with them.

Literature

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